

Isolation of Influenza A and B Viruses from Pigs at Bodija Abattoir, Ibadan, Nigeria

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Abstract: Both human and avian influenza virus can infect pigs and this make pigs to be intermediate hosts or mixing vessels for the generation of new influenza virus of pandemic potential, through genetic recombination. This study was designed to isolate and identify influenza virus strains from pigs at Bodija abattoir in Ibadan, Oyo State, Nigeria, to provide information on the activity of this virus in Ibadan. A total of 186 nasal swabs samples were collected from 93 animals over a period of two months from August to September, 2010. Isolation of influenza virus was carried out using conventional cell culture (MDCK) and ten to twelve-day old embryonated hen's eggs. Identification of isolates was carried out by haemagglutination-inhibition (HI) test using selected World Health Organization (WHO) influenza virus reference antisera. Out of 93 animals tested, 16 (17.2%) were positive for influenza virus infection. Identification of the isolates showed that there was co-circulation of different subtypes of influenza virus in pigs in Ibadan. The isolates were cross reactants of the H subtypes and influenza B virus. The isolation of influenza B, a virus that is known to infect only humans, from pigs suggests that the transmission of this virus from humans to pigs was occurring in Ibadan. Studies are in progress to determine the pathogenicity of this virus in pigs.

Key words: Bodija Abattoir • Haemagglutination-inhibition • Influenza A • Influenza B • Isolation • Pigs •

INTRODUCTION

Influenza is an infectious disease caused by a zoonotic virus capable of infecting humans, lower animals and birds. The virus belongs to the family of *Orthomyxoviridae*. They possess an eight-segment, negative sense, RNA genome which is approximately 13kb in size [1, 2]. Influenza is a disease of widespread occurrence and cause a significant problem in pigs throughout the world [3].

Swine are susceptible to infections with several different variants of the H1N1 and H3N2 subtypes of influenza A viruses. These include the viruses of classical swine, which is antigenically similar to virus isolated from avian sources and humans [4]. The H3N2 variants of human influenza viruses also replicate in swine. It has been postulated that pig is a "mixing vessel" where genetic reassortment between human and non-human influenza viruses can occur [4]. This makes it pertinent

that influenza surveillance in pigs should be carried out regularly [4]. Pigs play a pivotal role in the generation and transmission of avian influenza virus genus to human and have the potential to generate a new human pandemic strain. Pigs can be naturally or experimentally infected with avian and human influenza viruses because the epithelial cells in pig trachea contain both Neu Ac-2-3 Gal and Neu Ac-2,6 Gal receptors [5, 6] that are required by avian and human influenza viruses, respectively to initiate infection [7].

Influenza B viruses are only known to infect humans, while influenza C have been isolated only from humans, pig [8] and dogs [8].

Influenza A viruses cause swine influenza which is an acute, highly contagious respiratory disease of pigs, with classical aetiological types that include Influenza A subtypes H1N1, H1N2, H2N3, H3N1 and rarely Influenza C while Influenza B has not been reported in swine [8], but antibodies to influenza B has been detected in pigs [9, 3].

In Nigeria, influenza viruses have caused a significant amount of morbidity in the general human population but the incidence of excess mortality is unknown [10]. There is no sufficient information concerning various types of swine influenza viruses circulating in Nigeria [11]. This study therefore investigated the different types and subtypes of influenza viruses, which can cause possible intermittent infection in man, co-circulating among pig brought to Bodija abattoir in Ibadan, Nigeria.

MATERIALS AND METHODS

Study Site: Specimens were collected from pigs at Bodija abattoir which is a major abattoir located in Ibadan North Local Government Area of the city. The abattoir has existed for several decades with an average daily slaughter of 20-30 pigs. These pigs, reared as free range animals, were brought from different parts of the state.

Sampling Method and Specimen Collection: Using Fisher's formula for base simple calculation, 93 pigs were selected and 186 nasal swabs collected from them at the Bodija abattoir in Ibadan, Oyo State, South Western Nigeria from August to September 2010. The swabs were tested at the Department of Virology, College of Medicine, University of Ibadan. The health status of pigs tested is shown in Table 1. Two nasal swabs were obtained from apparently healthy, symptomatic and dead animals. The nasal swab was collected by inserting dry cotton tipped swab into the pig's snout and left in there for a few seconds. It was then slowly withdrawn with a rotational motion down the inside. The tip of the swab was cut into a vial containing 2mls of the two different viral transport media and the applicator stick was broken off.

The transport media used were PBS/ Glycerol and D-MEM. Antibiotics were added to prevent bacterial growth. The pH of 7.2-7.4 was maintained for the virus transport media.

The specimens in transport media were conveyed to the laboratory in a cold box lined with ice-packs, for virus isolation or for storage at -80°C temporarily.

Virus Isolation: Inoculation and harvesting procedure were performed in a class 2 biosafety cabinet. Each collected or stored specimen (0.2ml) was inoculated in duplicates into tissue culture tubes of a confluent monolayer of Madin-Darby Canine Kidney (MDCK) cell lines and incubated at 37°C. The incubated tubes were observed daily for CPE (Cytopathic Effects) for 7days. Cultures with that had CPE (3+ or 4+); supernatants from such tubes were stored in aliquots of 2ml at -80°C for

Table 1: Monthly status of pigs tested in the study

Animal Health Status	August	September	Total
AH	38	40	78
S	07	05	12
D	01	02	03
Total	46	47	93

Key AH-Apparently Healthy

S-Sick / Symptomatic

D-Dead

subsequent passaging. Blind passaging was also done for inoculated tubes that were not showing CPE. After the second passage that no CPE was observed they were regarded as negative. HA assay was carried out on supernatant that showed CPE using 0.5% chickens and 1% human O⁺ RBC at 4°C for 30 minutes.

The procedures involved in isolation in embryonated eggs were also performed in a class 2 biosafety cabinet. Ten to twelve day old embryonated chicken eggs were candled with the blunt side up, to select only the viable eggs were while the infertile, cracked, underdeveloped, dead or porous shelled eggs were discarded. After candling point of inoculation was marked on fertile embryonated selected eggs away from the course of blood vessels to prevent rupture. The surface of each egg was disinfected with 70% alcohol before punching a small hole on the shell over the air sac.

A 23G gauge needle was inserted into the amniotic sac and 100µl of the diluted specimen was injected into the amniotic cavity with the needle placed at an angle of 45° from the horizontal. The needle was withdrawn gently and additional 100µl was injected into the allantoic cavity. Two eggs were inoculated per specimen, while two eggs not inoculated from the same batch served as negative control. The hole in the shell was thoroughly sealed with wax, labeled appropriately with identification number. The eggs were incubated at 37°C for 3 days and checked daily for embryonic death. Eggs in which death occurred from 24hours post inoculation were immediately chilled at 4°C overnight or for 4 hours, before harvesting to prevent rupturing any blood vessel, while those without embryonic death were incubated for 72-96 hours before chilling and harvesting. The allantoic and amniotic fluids were then harvested with a sterile Pasteur pipette. Haemagglutination was performed according to World Health Organization (WHO) Manual on animal Influenza diagnosis and surveillance [12]. Haemagglutination assay was performed using the harvested fluids and incubated at 4°C for 30 minutes using 0.5% chicken RBC and 1% human O⁺ RBC. If no HA was present, the specimen was passaged two or more times and HA repeated before regarding the samples as negative.

Identification of Isolates: The haemagglutination inhibition assay (HI) was used for isolate identification. This procedure was carried out according to the manual on animal influenza diagnosis and surveillance [12]. The reference antisera used include:

- Influenza A (H1), Centres for Disease Control and Prevention Control (CDC) reference antiserum
- Influenza A (H3), Centres for Disease Control and Prevention Control (CDC) reference antiserum
- Pandemic Influenza (H1N1), Centres for Disease Control and Prevention Control (CDC) reference antiserum
- Influenza B, Centres for Disease Control and Prevention Control (CDC) reference antiserum

The control antigen used was:

- Influenza A (H1), Centres for Disease Control and Prevention Control (CDC) reference antigen

Our tests were confined to the above Influenza reference antisera and antigen because of the difficulty encountered in obtaining a complete influenza virus identification panel.

The test sera was treated with receptor destroying enzyme (RDE), by adding 3 volumes of RDE to 1 volume of serum and the mixture was incubated in a water bath at 37°C overnight.

The reference antisera were heat-inactivated in a 56°C water bath for 30 minutes to inactivate remaining RDE. Sera dilution was made at 1:10 with physiological saline 0.85% NaCl after being cool. Removal of natural serum agglutinin from the reference treated sera (diluted) was done by adding 0.1ml packed washed erythrocytes per 1ml to diluents serum. Incubated for 30 minutes at room

temperature, occasional mixing was done. The treated sera were centrifuged at 1,200rpm for 10 minutes to retain the serum. Carefully the adsorbed serum was removed without disturbing the packed cells with aid of a sterile pipette. The HI procedure was conducted as follows: Dilutions which contain 4HA units / 25µl of isolates or control antigens against 0.5% chicken RBC suspension were obtained and back titration was also conducted.

The rows of wells in a V-bottom microtitre plate were labeled for each isolate and positive control (reference antigen). The last 2 columns were used as RBC control wells and negative control (uninfected allantoic fluid). Plates were shaken and incubated at room temperature for 30 minutes. Thereafter 25µl of 0.5% chicken RBC was added to all wells and plate agitated manually and incubated at room temperature for 30 minutes.

RESULTS

The CPE observed was characterized by cell rounding and aggregation of cells into grape-like clusters 3-4 days post inoculation. Out of 46 samples inoculated into MDCK cell line, 1 (4.34%) produced CPE using Glycerol/PBS as VTM, 3 (13.04%) produced CPE using D-MEM as VTM as shown in Table 2.

A total of 19 isolates that haemagglutinated chicken and human O⁺ erythrocytes 1 (5.26%) from MDCK and 18 (94.74%) from embryonated eggs and were all subjected to HAI test.

Isolates from Glycerol/PBS samples had 8 (8.6%) for HI, 7 (7.5%) for H3, 7 (7.5%) for H1N1 and 7 (7.5%) for influenza B as shown in Table 3.

Isolates from D-MEM samples had 8 (8.6%) for HI, 7 (7.5%) for H3, 7 (7.5%) for H1N1 and 8 (8.6%) for influenza B as shown in Table 3. There was cross-reactivity of the reference antisera with the isolates tested.

Table 2: Summation of CPE in MDCK with different VTM

VTM	No of inoculated samples	No (%) of sample (s) showing CPE	No (%) of samples not showing CPE
Glycerol / PBS	23	1 (4.34)	22 (95.65)
D-MEM	23	3 (13.04)	20 (86.95)
Total	46	4 (8.70)	42 (91.30)

Table 3: Summation of HAI test with different VTM isolates

VTM	No (%) of isolates for H1	No (%) of isolates for H3	No (%) of isolates for H1N1	No (%) of isolates for Influenza B
Glycerol / PBS	8 (8.6)	7 (7.5)	7 (7.5)	7 (7.5)
D-MEM	8 (8.6)	7 (7.5)	7 (7.5)	8 (8.6)
Total	16 (8.60)	14 (7.53)	14 (7.53)	15 (7.97)

Table 4: Monthly animal health status distribution of influenza virus isolated

Animal health status	August		September		Grand total	
	No of animal sampled	No (%) of isolate (s)	No of animal sampled	No (%) of isolate (s)	No of animal sampled	No (%) of isolate (s)
AH	38	9 (23.68)	40	3 (7.5)	78	12 (12.90)
S	07	3 (42.86)	05	1 (20)	12	4 (4.30)
D	01	0 (0)	02	0 (0)	03	0 (0)
Total	46	12(12.90)	47	4 (4.30)	93	16 (17.20)

Key: AH = Apparently Healthy; S-Sick/ Symptomatic; D-Dead

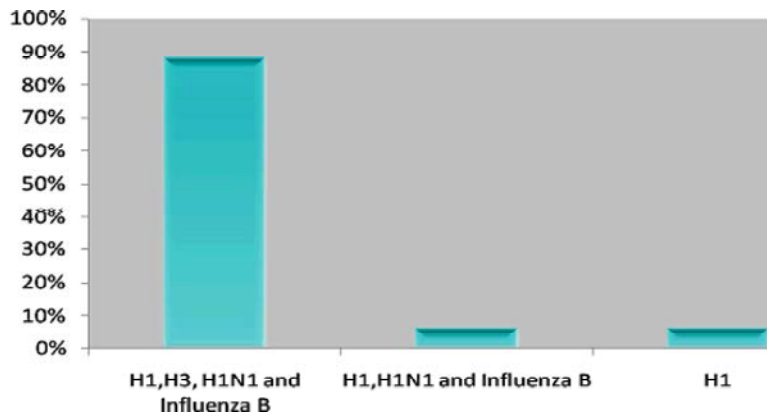


Fig. 1: Frequency of Isolates Tested Against The WHO Reference Antisera for Identification

With the four reference antisera 14 (88%) isolates reacted with the four (H1, H3, H1N1 and influenza B), 1 (6%) reacted with three reference antisera (H1, H1NI and influenza B) and 1 (6%) also reacted with one reference antiserum (H1) as shown in Figure 1.

A total number of 186 samples were processed for virus isolation, 92 samples for the month of August and 94 samples for the month of September. Influenza isolates were obtained for the duration of the study.

In the month of August, 46 animals were sampled, out of which 38 were apparently healthy, 7 were sick and 1 dead. A total number of 12 (12.90%) swine influenza viruses were isolated. 9 (23.68%) from apparently healthy animals, 3 (42.86%) and 0 from dead animal.

In the month of September, 47 animals were sampled out of which 40 were apparently healthy, 5 were sick and 2 dead. A total number of 4 swine influenza viruses were isolated 3 (7.5%) from apparently healthy animals, 1(20.07) from sick and 0 from dead animals as shown in Table 4. For the period of study 16(8.60%) HI isolates, 14 (7.58%) H3 isolates, 14(7.55%) of H1N1 and 15 (7.97%) were obtained as shown in Table 4. Influenza B isolates were also obtained at the end of HAI test as shown in Table 4.

DISCUSSION

In recent years, swine influenza virus has become wide spread in Nigerian pigs and there could be sporadic infections in man from this source [11]. In the study the authors used two viral culture methods which were the MDCK and fertile eggs. Both methods are known to be sensitive for influenza isolation [12]. However, the MDCK used in this study was found not to be as sensitive as it should be because of the passage number. Therefore in the result, embryonated fertile eggs were considered reliable than that in MDCK cell culture.

The prevalence of influenza virus in swine population has been demonstrated by researchers in Nigeria [11], serological surveillance studies revealed that prevailing human H1N1 are readily transmitted to pigs [3]. Human influenza A (H3N2) viruses have been isolated from pigs [6].

The findings of this study suggest that different subtypes of Influenza A virus H1, H3 and pandemic H1N1, co-circulated among pigs in Ibadan during this period. As a result of this there could be reassortant strains in the pigs sampled, which could be transmitted to the handlers and get infected with the reassortant strains which could be of pandemic potential and this variant could circulate around the globe in successive waves.

Most of the pigs for this surveillance were pigs reared in free range that have daily interactions with both human and fowls. This interaction could have led to the reassortment of the virus. This reassortant strain could also have the ability to cross host-specific barriers. This makes pig an important vessel in which influenza surveillance should be carried out regularly to prevent the spread of new pandemic influenza viruses.

Influenza occurs most often in Nigeria during the harmattan period with a peak in rainy season [5,13].

In the month of August 12 isolates of swine influenza were obtained whereas 4 isolates of swine influenza for September. The result of previous, [5, 13] and present study indicates that at the beginning of rainy season up till August is the period when influenza virus activity is the highest in Nigeria. The co-circulation of several subtypes (H1, H3, H1N1 and influenza B) of virus in this study provides further evidence that pigs serve as intermediate host or mixing vessels and emphasizes on the importance of reinforcing swine influenza virus surveillance in Nigeria.

From the study, the highest numbers of swine influenza isolates 12/78 were obtained from the apparently healthy animals. Therefore this group of animals should be included among the population for sampling during influenza surveillance. Although swine influenza isolates were obtained from sick animals, the prevalence was low. None of the specimens from dead animals yielded swine influenza isolates. It is possible that the animals died of infections other than swine influenza as no isolate was obtained from the dead pigs. It is also possible that the infectivity of the virus would have been lost in the nasal secretions of the dead animals before arrival at the abattoir. If indeed they died of influenza.

Although influenza B and C viruses mainly infect humans. Influenza B viruses have been isolated from horse, pig and dog [14]. In this study, influenza B virus was isolated from Nigeria pigs, suggesting that infection of pigs by influenza B virus may be common than originally thought. In recent years, Influenza B virus has tended to be prominent every 2-3 years. The interplay between Influenza viruses and their hosts may influence their impact and evolution [15]. It was suggested by Daniel and Carolyn, 2003, [16] that the source of influenza B infections to pigs may be human as pigs sampled in this study had interactions with human because they were reared on free range system.

Further evidence of influenza B virus infections in pigs was provided by Brown, *et al.*, 1995 [17] who reported that seven pigs were seropositive by

haemagglutination inhibition, virus neutralization and immune blotting assays for antibody to influenza B. In sero surveillance studies in Hungary there were seropositive pigs in test to influenza B virus following a human epidemic. Experimental transmission of influenza B virus to young pigs suggested that pigs are susceptible to infection [18].

All these studies provide further evidence of influenza B virus infection in pigs. It has been suggested that transmission of influenza from human to pigs occur, but infection fail to spread [3]. This may explain the lack of reports of influenza B virus epizootics in pigs. It could be possible that those isolates are recombinants isolates that are reacting with influenza B reference antiserum, further molecular studies are in progress to ascertain the influenza isolates and its' pathogenicity.

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